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Synergistic antimicrobial activity of black seed (*Nigella sativa*) with ciprofloxacin against multidrug resistant *Salmonella typhimurium*

Fazal-ur-Rehman[1,2], Abdul Hannan[2], Fiaz Ahmad[1,3,4], Maryam Khan[1], Saba Shamim[1*]

[1]*Institute of Molecular Biology and Biotechnology (IMBB), The University of Lahore, Defence Road Campus, Lahore, Pakistan*

[2]*University of Health Sciences, Lahore, Pakistan*

[3]*Shaikh Zayed Hospital, Lahore, Pakistan*

[4]*Shalamar Hospital Lahore, Pakistan*

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Abstract

Typhoid fever is an important cause of disease and death in south east and south central Asia. Itis becoming resistant to more potent drugs such as fluoroquinolones and third generation cephalosporins. It was a descriptive experimental study in which preserved bacterial isolates of multi drug resistant (MDR) *Salmonella typhimuirum* (n=49) were used. The methods used for antibacterial effect was agar well diffusion method and disc diffusion method for black seed extract and ciprofloxacin respectively, agar dilution method for minimum inhibitory concentration (MIC), and microtitre checkerboard method for synergism. The antibacterial effect of ethanolic black seed extract by agar well diffusion method did not show any inhibition zone at 100 % concentration v/v. By agar dilution method, the MIC of ciprofloxacin was 0.25 µg/ml, 0.5 µg/ml and 1 µg/ml for 6 isolates (12 %), 2 µg/ml for 20 isolates (40 %), 4 µg/ml for 48 isolates (97 %), 8 µg/ml for 49 isolates (100 %). The extract inhibited 23 isolates (46 %) at 64 % concentration which was the highest concentration used by agar dilution method. The ciprofloxacin and ethanolic black seed extract exhibited synergism against two *S. typhimurium* isolates, indifference against 45 tested isolates, and not determinable against 2 isolates. A newly tried combination showed to be synergistic against two isolates, indifferent against 45 tested MDR *S. typhimurium* isolates and not determinable (ND) against 2 isolates.

* **Corresponding Author:** Saba Shamim ✉ saba.shamim@imbb.uol.edu.pk

Introduction

Typhoid is a disease caused by the bacterium *Salmonella enterica* serovar typhi (*S. typhimurium*). It is a serious and occasionally fatal disease which spreads via oro-fecal route. In developing countries, it represents a significant health problem. Almost 12 million cases of typhoid occur worldwide each year causing 128,000 deaths (Neuzil *et al.*, 2019). Typhoid fatality rate in the pre-antibiotic era was up to 20% which was dropped to less than 1% due to treatment by antimicrobial agents (Manchanda *et al.*, 2006). Chloramphenicol was introduced in 1948 for typhoid treatment. But it developed resistance against it by mid 1950s (Cooke and Wain, 2004). During the 1960s, resistance to three or more first-line antibiotics including ampicillin, trimethoprim-sulfamethoxazole, and chloramphenicol, known as MDR, became more common, and MDR *S. typhimurium* began to be reported more frequently. The use of ciprofloxacin was advocated as it was reported to be very potent. However, within a few years, it showed significant resistance to quinolones (nalidixic acid) and medium level of resistance to ofloxacin (Dyson *et al.*, 2019).

The emergence of *S. typhimurium* strains resistant to nalidixic acid with decreased susceptibility to ciprofloxacin had further complicated the situation and questioned the efficacy of fluoroquinolones (Kasper *et al.*, 2010). So, the third generation cephalosporins have remained to be the likely choice in its treatment (Harish *et al.*, 2008). But these substitute regimens have many disadvantages, like highly expensive, parenteral route and long defervescence time and the treatment of typhoid is more over complicated due to the renitency developed against the extended spectrum cephalosporins like ceftriaxone, cefipime and cefixime (Capoor and Nair, 2010). Currently, a point is reached where many antibiotics are no more effective against even the simple infections. Therefore, there is an urgent need to address this issue and to develop new and useful antibiotics to escape the pre antibiotic era (Marten and Demain, 2017).

To overcome it, plants are considered an effective substitute as antibacterial agents (Wikaningtyas and Sukandar, 2016). There is increasing evidence about medicinal plants having synergistic and side effects neutralizing combinations (Gilani, 2005). *N. sativa* seeds have a very important place in Islamic world as it is a common drug used in Tibb e Nabawi and the Prophet Muhammad (PBUH) stated that black seed is cure for every disease except death (Sahih Bukhari, 7:591). The Dioscorides, a Greek herbalist, discussed *N. sativa* in the third volume of his Meteria Medica in the first century A.D. and recommended black seed to be used as a remedie for colds, warts, headaches, toothaches, swellings, dyspnoea and spider bites (Yarnell and Abascal, 2011). *N. sativa*, belongs to the botanical family of Ranunculaceae, commonly known as black seed. It grows in the Middle East, Eastern Europe and Western Asia (Tavakkoli *et al.*, 2017). They are a source of active ingredients (Salem, 2005). Its extract as well as oil have been reported to have antidiabetic, anti-inflammatory, antitumor, antiallergic, antifungal, antibacterial, antiviral as well as a relieving agent for headache, cough and abdominal pain (Develi *et al.*, 2014). A study from Pakistan showed its *in vitro* antibacterial effect against the MRSA by inhibiting all strains (Hannan *et al.*, 2008). In two studies from Sudan and India, antibacterial and antifungal effects were studied (Kabbashi *et al.*, 2015). Hosseinzadeh *et al.* (2007) reported its antibacterial effect against both Gram positive and Gram negative organisms. A very limited data is available on synergistic effect of *N. sativa* with ciprofloxacin. Therefore, this study was an attempt to evaluate the synergistic antimicrobial activity of black seed with ciprofloxacin against the isolates of multidrug resistant *S. typhimurium*.

Materials and methods

Study design

It was a descriptive experimental study. Preserved bacterial isolates of multidrug resistant (MDR) *Salmonella typhimurium* (n=49) were obtained. Revived isolates were stored in microbank at -70 °C. One ATCC (American Type Culture Collection) standard strain, *Salmonella paratyphi* A (ATCC

9150) was used as a reference strain to ensure the quality control.

Inclusion criteria

Multidrug resistant (MDR) i.e. (ampicillin, chloramphenicol and co-trimoxazole) resistant and ciprofloxacin susceptible isolates were used for this study.

Preparation of ethanolic black seed extract

Indigenous black seeds were procured from the market. Before grinding the seeds, it was checked that seeds were free of any dust. They were placed in a clean bottle in which ethanol was added. After four days, the filtrate was filtered and evaporated using rotary evaporator. The extract was collected in a vial and placed in refrigerator till experimentation (Hosseinzadeh *et al.*, 2013).

Ciprofloxacin base powder

It was purchased from the market.

Antimicrobial susceptibility of Salmonella typhimurium isolates

The antimicrobial susceptibility to ciprofloxacin was determined (CLSI, 2015) using commercially available 5 µg ciprofloxacin disc (Oxoid, UK).

Confirmation of Salmonella typhimurium as MDR

The *S. typhimurium* isolates were confirmed as MDR using CLSI guidelines and employing commercially available discs of chloramphenicol (30 µg), ampicillin (10 µg) and co-trimoxazole (25 µg) (Oxoid, UK).

Screening for inhibitory effect of ethanolic black seed extract

The method of Lee *et al.* (2011) was followed to determine the antibacterial activity of black seed extract. Ethanolic black seed extract and controls were tested in triplicate by adding 75 µl of each concentration with the allocated number on the Petri dishes (four wells per plate). Initially 100 % extract with an equal quantity of diluents i.e. distilled water as negative control and ceftriaxone as positive control were run. The procedure was repeated with different concentrations (50 %,100 %) of the extract. After application, the plates were incubated over night at 37 °C. The diameters of the clean zones were measured in mm with digital caliper (Sylvac, Fowler, and ultra-call 11).

Minimum Inhibitory Concentration (MIC)

It was determined by the agar dilution technique according to the CLSI document (M100-S25 2015). The concentrations of ciprofloxacin and extract used were from 0.25 µg/ml to 32 µg/ml and 2 % to 64 % respectively.

Synergy testing by checkerboard titration technique

Broth microdilution checker board technique was employed to evaluate the activity of specified concentrations of antimicrobial agents in combination for 18-24 hours using microtitre plates (Visalli *et al.*, 1998; Veloira *et al.*, 2003). The activity of antimicrobial ciprofloxacin and black seed extract combination was evaluated against isolated strains of MDR *S. typhimurium*. The *in vitro* activity of antimicrobial combinations was calculated mathematically as fractional inhibitory concentration index (Σ FIC). This was equal to the sum of the FICs for individual drugs in combination.

The FIC for a drug was defined as the minimal inhibitory concentration (MIC) of the drug in combination divided by the individual MIC of the drug. ΣFIC_{min} for a drug combination is the minimum value of ΣFIC that inhibited organism while the ΣFIC_{max} for a drug combination is the highest value of ΣFIC which inhibited the organism.

Inoculum preparation

The turbidity of the bacterial suspension was adjusted to achieve a density equivalent to the turbidity standard of 0.5 McFarland (approx. 1.5×10^8 CFU/ml of suspension).The suspension was further diluted 1:100 by adding 0.1 ml of suspension to 9.9 ml cation–adjusted Mueller Hinton Broth (CA-MHB) to achieve the final inoculum containing approx. 1.5×10^6 CFU/ml of the suspension. The inoculum was used within 15 minutes of preparation.

Preparation of stocks and working solutions

The stock solutions and serial two-fold dilutions of each drug were prepared according to the recommendations of NCCLS immediately prior to testing by applying the formula $C_1V_1 = C_2V_2$.

Preparation of checkerboard microdilution panels

Eight rows and eight columns of microtitre plates were dispensed with antimicrobial solutions to generate checkerboard. The drug A ciprofloxacin was used along the columns of the plates. The drug B i.e. black seed extract was used along the rows of the plates. For each strain, one microtitre plate was prepared. About 50 µl of Mueller Hinton Broth (MHB) was added to each well from column 1 to 9 and from rows A to H. The working stock of ciprofloxacin was added with a 50 µl in each well of column A2 to A9 by increasing the concentration. The working stocks were two times higher than the desired concentration in each well of the microtitre plate. The concentration of ciprofloxacin in column A2 to H2 was 0.125 µg/ml and so on increased the concentrations in the next column till column 9. The black seed extract was inoculated in rows with increasing concentrations through rows i.e. from B1 to B9 was the concentration of 4 % and so on increasing in the next rows till row H1 to H9. A1 and H12 were sterility control by adding only 100 µl Mueller Hinton Broth. A12 was organism control. The final volume of broth after addition of both drugs in each well was 100 µl. The 50 µl MHB was added in A2 to A9 and B1to H1. A12 was organism control by adding 100 µl of suspension. In this way, all possible combinations were made. The plates were labelled, sealed and stored overnight at -20 ± 2 °C.

Inoculation and incubation of microtitre plates

The plates were thawed and used immediately. The 10 µl of diluted bacterial inoculum (10^6 CFU/ml) was added in each well of the plate except from A2 to A9 and B1 to B9 up to H1 to H9 except A1 and H12 which are broth sterility control. Since each well contained 100 µl of antimicrobial solution so the inoculum further diluted 10 times (1:10) thereby resulting in approx. 10^5 CFU/ml. The inoculated plates were incubated overnight.

Purity plate

Using a 0.001 ml calibrated wire loop, 0.001 ml of inoculums was taken from bacterial suspension tube and inoculated on blood agar plate for isolation and incubated at 35±2 °C for 18 hours. This was a purity plate.

Inoculum count verification plate

Immediately after inoculation, 10 µl was transferred from growth control well into 1 ml of sterile normal saline and mixed well. A 10 µl from this diluted suspension was inoculated on blood agar plate by streaking in several directions with a loop and incubated at 35±2 °C for 18 hours. This was to observe colony count.

Reading of microtitre plates

Purity and inoculums count verification plates were examined for purity and satisfactory count respectively. Purity plate showed no “mixed growth” while inoculum verification plate showed colonies between 50 and 80. In addition, well A12 was growth control and showed heavy turbidity and A1 and H12 were sterility control and showed no growth and these wells were taken as reference for no growth. In each plate, row A was examined for no growth to determine the MIC of ciprofloxacin. In each plate, column 1 was examined for no growth, as indicated by absence of turbidity, to determine MIC of second drug i.e. black seed extract. After that each combination well was examined for growth and the well with no growth was recorded as the FIC.

Interpretation of the results

The calculations for Sigma Fractional Inhibitory concentrations (ΣFICs) are given as:

ΣFIC = FIC A + FIC B

Here

FIC A = MIC of drug A in the combination ÷ MIC of drug A alone

FIC B = MIC of drug B in the combination ÷ MIC of drug B alone

FIC index was taken as summation of individual FICs given as:

Σ FIC = FIC A + FIC B

Here combination interactions were interpreted on the basis of calculated values of FIC index (ΣFIC) (Visalli *et al.*, 1998; Veloira *et al.*, 2003) as follows:

Synergism = Σ FIC is ≤ 0.5

Indifference = Σ FIC is > 0.5 and ≤4.0

Antagonism = Σ FIC is > 4.0

Results

(Figures and their numbers deleted)

The antibacterial effect of ethanolic black seed extract by agar well diffusion method did not show any inhibition zone at 100 % concentration (v/v). Table 1 and Table 2 shows the percentage of MDR *S. typhimurium* isolates that were inhibited at different concentrations of ciprofloxacin and ethanolic black seed extract by agar dilution method respectively. Six isolates (12 %) were inhibited at 0.25 µg/ml, 0.5 µg/ml and 1 µg/ml of ciprofloxacin, 20 isolates (40 %) were inhibited at 2 µg/ml, 48 isolates (97 %) were inhibited at 4 µg/ml, 49 isolates (100 %) were inhibited at 8 µg/ml. 23 isolates (46 %) were inhibited at 64 % concentration of the extract which is the highest concentration used (Table 1). Table 2 show the different concentrations of ethanolic black seed extract used in agar dilution method.

Table 1. Percentage of MDR *S. typhimurium* isolates inhibited at different concentrations of ciprofloxacin (n=49).

MIC of ciprofloxacin (µg/ml)	Percentage of MDR *S. typhimurium* isolates inhibited
32	100 % (49 isolates)
16	100 % (49 isolates)
8	100 % (49 isolates)
4	97 % (48 isolates)
2	40 % (20 isolates)
1	12 % (6 isolates)
0.5	12 % (6 isolates)
0.25	12 % (6 isolates)

The concentrations used were from 64 % the highest to 2 % the lowest. Only 23 isolates (46 %) of MDR *S. typhimuirum* isolates were inhibited at 64 % concentration of the extract which is the highest concentration used. The remaining concentrations did not show any inhibitory effect (Table 2). MIC ranges of ciprofloxacin and clack seed extract are shown in Table 3. The MIC_{50}, MIC_{90} and MIC_{100} of the extract was >64 % which was the highest concentration of the extract used. MIC_{50} of ciprofloxacin was >2 µg/ml and MIC_{90} and MIC_{100} of Ciprofloxacin was 4 µg/ml and 8 µg/ml respectively.

Table 2. Percentage of MDR *S. typhimurium* isolates inhibited at different concentrations of extract (n=49).

MIC of extract (%)	Percentage of isolates of MDR *S. typhimurium* inhibited
64 %	46 % (23 isolates)
32 %	0
16 %	0
8 %	0
4 %	0
2 %	0

Table 4 is showing FICIs of ciprofloxacin and extract combination for 49 tested isolates of MDR *S. typhimurium*. The 2 strains showed growth with highest concentration of the extract i.e. 256 %, so the results were not determinable for these strains. Table 4 is showing FICIs of ciprofloxacin and EBSE combination for 49 tested isolates of MDR *S. typhimurium*. The data shows that ciprofloxacin and

ethanolic black seed extract (EBSE) have exhibited synergism against two *S. typhimurium* isolates, indifference against 45 tested isolates, and not determinable against 2 isolates. These 2 strains were not determinable because the highest concentration of BSE i.e. 256 % did not inhibit these strains (Table 5). Hence our newly tried combination showed to be synergistic against two isolates, indifferent against 45 tested MDR *S. typhimurium* isolates and not determinable (ND) against 2 isolates.

Table 3. MIC of ciprofloxacin and ethanolic black seed extract against MDR *S. typhimurium* isolates.

Isolates	Antimicrobial agent	MIC			
MDR *S. typhimurium* (n=49)		MIC range	MIC_{50}	MIC_{90}	MIC_{100}
	Ethanolic black seed extract	2 %-64 %	>64 %	>64	>64
	Ciprofloxacin	0.25-32 µg/ml	> 2 µg/ml	4 µg/ml	8 µg/ml

MIC= minimum inhibitory concentration.

Discussion

(Unnecessary paragraphs are deleted according to the reviewer's suggestion)

The present study included 49 strains of MDR *S. typhimurium*. The ciprofloxacin susceptibility was performed and all the strains were susceptible to ciprofloxacin according to CLSI, (2015). The MIC of ciprofloxacin was determined. The MIC range of ciprofloxacin was 0.25-32 µg/ml. The synergism of both agents was investigated by microtitre checkerboard method. The concentrations of ciprofloxacin and BSE in checkerboard method were 0.125-16 µg/ml and 4 % to 256 % respectively. Two strains AMC 7 and AMC 26 were picked up at random and were evaluated for their susceptibility to the extract. No zone of inhibition was observed at 100 % v/v concentration of extract. MIC of the extract was performed by agar dilution method. The results showed 23 isolates (46 %) were inhibited at the 64 % v/v concentration of the extract. ATCC 9150 *Salmonella paratyphi* A also showed susceptibility at 64 % v/v of the extract. In agar well diffusion, the extract did not show any antibacterial activity even at 100 % of extract, however by agar dilution method the antibacterial activity was noted. In agar dilution method, the extract is directly incorporated into the medium, therefore bacteria are brought into direct contact with all components of the extract immediately on application to the agar rather than by agar well diffusion method in which it will be difficult for some components of the extract to diffuse through the agar (Lusby *et al.*, 2005). The agar well diffusion method may need more time to produce any possible effect (Islam *et al.*, 2013).

Many studies have shown that the ethanolic extracts of *N. sativa* failed to show any effect against many Gram positive and Gram negative organisms (Dulger and Gonuz, 2004; Ababutain, 2011). Our study showed that by agar well diffusion method there was no inhibitory zone produced and by agar dilution method the MIC of 46 % of the strains was at the highest 64 % concentration. In a study the antibacterial effect of the essential oil of *N. sativa* was tested by disc diffusion method.

Table 4. FIC indices of cipro-extract combination against MDR *S. typhimurium* isolates (n= 49).

No. of isolates	FICI
2	≤ 0.5
1	0.51
1	0.56
1	0.63
2	0.75
12	1.01
2	1.03
17	1.06
6	1.13
1	2.01
2	2.06

FICI= fractional inhibitory concentration index.

The concentration range used was 1/50 to 1/4000 v/v. All the microorganisms were inhibited at different concentrations but *S. typhimurium* showed

resistance to essential oil at all concentrations (Amina and Rachida, 2013). According to other researchers, methanolic extracts of 306 plants including *N. sativa* total parts were screened for antimicrobial activity against eight different pathogens including *S. typhimurium,* but only *B. subtilis* and *S. aureus* showed to be sensitive against *N. sativa* (Ababutain, 2011; Ali *et al*., 2011). Kamal *et al*., (2010) was of view that there might be some changes in the results if they would have used other extracts.

The strains they tested were MDR, so it might also be the reason that inhibitory zone was not produced by agar well diffusion method and showed MIC at highest concentration against some isolates by agar dilution method. Combination of one drug with a natural antimicrobial agent is considered as an interesting approach. The use of pure natural compounds or plant extracts in combination with regular antibiotics may hold pronounced promise for rapidly contributing affordable treatment options. Actually, some combinational antibiotic therapies are already available clinically (Cheesman *et al*., 2017). In this study, the effective treatment against MDR *S. typhimurium* was studied employing a combination of ciprofloxacin and black seed extract.

There are many studies in which plant extracts combined with drugs have been evaluated for their activity (Aburjai *et al*., 2001; Siriwong *et al*., 2016; Ali *et al*., 2007). A study has shown the synergistic effect of the two main components thymoquinone (TQ) and thymohydroquinone (THQ) of *N. sativa* essential oil in combination with different antibiotics such as ampicillin, chloramphenicol, gentamicin, tetracycline and ciprofloxacin against *S. aureus*, but against Gram negative bacteria their synergism, antagonism and indifference were detected in 28.9 %, 23.6 % and 47.5 % of the tested combinations respectively (Halawani, 2009). In a study, the MIC and synergism of *N. sativa* with honey was evaluated by using agar dilution method against *P. aeruginosa*.

Table 5. Checkerboard panel (Rows and columns title Edited).

Drug A (Ciprofloxacin)	Drug B (black seed extract)											
	1	2	3	4	5	6	7	8	9	10	11	12
A	Sterility control	0.125	0.25	0.5	1	2	4	8	16			
B	4 %	0.125 4	0.25 4	0.5 4	1 4	2 4	4 4	8 4	16 4			
C	8 %	0.125 8	0.25 8	0.5 8	1 8	2 8	4 8	8 8	16 8			
D	16 %	0.125 16	0.25 16	0.5 16	1 16	2 16	4 16	8 16	16 16			
E	32 %	0.125 32	0.25 32	0.5 32	1 32	2 32	4 32	8 32	16 32			
F	64 %	0.125 64	0.25 64	0.5 64	1 64	2 64	4 64	8 64	16 64			
G	128 %	0.125 128	0.25 128	0.5 128	1 128	2 128	4 128	8 128	16 128			
H	256 %	0.125 256	0.25 256	0.5 256	1 256	2 256	4 256	8 256	16 256			

The combination showed synergism and MIC was decreased (Abdelmalik *et al*., 2012). Looking at many studies which showed the synergistic effect of black seed with different drugs and natural products, the idea was taken to test the combination. Our study showed synergism against two isolates i.e. AFIP 6 and AFIP 10, indifference against 45 isolates and not determinable (ND) against two strains. Our results were in agreement with the previous findings (Zhi-Qing *et al*., 2002; Halawani, 2009). For the assessment of synergism many methods including time kill assays, checker board titration technique, disc diffusion, etc. have been used in different studies. Currently MIC was determined by agar dilution method whereas checkerboard method helped in the determination of synergism. There were differences in the results of the tested combinations (Mayer and Nagy, 1999). Our tested combination for synergism might have shown different results if evaluated by other two methods.

As our research has shown synergism against 2 isolates, indifference against 45 isolates and not determinable (ND) against 2 isolates, the combination can be tried in vivo and in vitro using other extracts. The MIC of ciprofloxacin and ethanolic

black seed extract in combination would have expected to be notably reduced as compared to the MICs of both individually. This could lead to reduction in dose requirement of ciprofloxacin in typhoid patients and could be a solution to treatment failures with fluoroquinolones. However, the hypothesis could not be proved to a good extent.

The limitations of this study are; black seed was used in the form of crude ethanolic extract. There might be a possibility that thymoquinone, thymohydroquinone and other active antibacterial components concentration have been reduced during ethanolic extract preparation and only ethanolic extract was used.

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